EUROPEAN RESPIRATORY journal

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Research letter

Breath volatile organic compounds and inflammatory markers in adult asthma patients – negative results from the ALLIANCE cohort

Olaf Holz, Benjamin Waschki, Henrik Watz, Anne Kirsten, Mustafa Abdo, Frauke Pedersen, Markus Weckmann, Oliver Fuchs, Anna-Maria Dittrich, Gesine Hansen, Matthias V. Kopp, Erika v. Mutius, Klaus F. Rabe, Jens M. Hohlfeld, Thomas Bahmer, the ALLIANCE Study Group

Please cite this article as: Holz O, Waschki B, Watz H, *et al*. Breath volatile organic compounds and inflammatory markers in adult asthma patients – negative results from the ALLIANCE cohort. *Eur Respir J* 2020; in press (https://doi.org/10.1183/13993003.02127-2020).

Breath volatile organic compounds and inflammatory markers in adult asthma patients – negative results from the ALLIANCE cohort

To the editor:

Breathomics in asthma is a rapidly growing area of significant scientific interest, as indicated by a recently published review, two research articles, and their accompanying editorials in high impact pneumology journals [1–5]. The repeatedly observed associations between breath volatile organic compounds (VOCs) and sputum or blood inflammatory cells [2,3] suggest that *breathomics* are on the brink of introduction as a valuable clinically tool. However, there are also major concerns about unresolved methodological issues and a general paucity of high-quality data [1,6]. In this letter we detail our concerns with *breath-omics* based on data from a cohort of adult asthma patients with a broad spectrum of clinical phenotypes.

In the adult arm of the ALLIANCE asthma cohort we prospectively recruited patients with an established diagnosis of asthma [7] across different severity grades and inflammatory phenotypes as identified by FeNO, blood and sputum differential cell counts [8]. As recently recommended [5], the patients were clinically well characterized, the asthma diagnosis was thoroughly established, and breath VOCs were compared between different asthma phenotypes.

For the analysis we included 133 adult patients that attended their 12-month follow-up visit from 2015 to 2016 at LungenClinic Grosshansdorf. To examine patients under stable conditions, all visits were scheduled at least four weeks after an acute severe exacerbation (defined as oral steroid burst therapy for at least three consecutive days) or asthma-related hospitalization. The study (NCT02419274) was approved (Medical School Luebeck ethics committee, Az.12-215) and all participants gave their written informed consent before inclusion.

The patients were grouped into five clinically established phenotypes according to disease severity (2014 ERS/ATS guidelines [9]), type 2 airway inflammation (blood eosinophils ≥300/µl), and smoking status (Panel-figure 1.A). We performed spirometry, body-plethysmography, impulse-oscillometry, and measured FeNO [8]. Blood differential cell counts and induced sputum [10] were assessed by established protocols. The collection and analysis of breath VOCs is described in detail in [11]. Patients inhaled active-carbon filtered

room air and exhaled into an aluminum reservoir tube to avoid the use of sampling bags. During 5min collection, 2.5L breath were loaded onto each of two Tenax®TA adsorption tubes, which were analyzed by gas-chromatography/mass-spectrometry (GC/MS). 134 VOCs were assessed in total (listed in Table 2 of [11]). Fourty VOCs were excluded because in at least in 85% of the study participants the values were below the limit of detection. VOC data was log-transformed prior to analysis.

The validity and plausibility of the VOC data is supported by several aspects and observations. (I) The method used for collection and analysis was benchmarked in the Peppermint oil trial [12,13], in which we demonstrated washout kinetics of peppermint oil compounds after ingestion of a respective capsule. (II) The two simultaneously collected adsorption tubes in this study showed a very close agreement (median $r>0.87$). (III) As expected, acetone and isoprene were the most abundant VOCs in breath, while cleaning and disinfectant related VOCs like propanol-1, propanol-2 and ethanol were predominantly found in room air. (IV) We found highly significant differences for smoking related VOCs like acetonitrile, benzene and cyclohexadien ($p<0.001$, respectively) between active smokers and non-smokers (Panel-figure 1.A). In addition, these compounds indicated that five patients potentially misjudged their smoking behavior or experienced a substantial passive smoke exposure. (V) In line with others [14], we found higher levels of isoprene in male subjects ($p<0.001$).

Patients' characteristics according to asthma phenotypes are shown in panel-figure 1.A. In contrast to others, we observed no statistically significant correlations between breath VOC levels and markers of inflammation like sputum eosinophils, blood eosinophils, sputum neutrophils or exhaled NO, after adjusting the p-values for multiple testing using the Benjamini-Hochberg method [15]. The histograms of all unadjusted p-values of the correlations between VOCs and markers of inflammation are shown in panel-figure 1.G. We tested these correlations also within the subgroups of patients with comparable results. Panel-figures 1.C-F show the correlations between sputum neutrophils and eosinophils for three markers suggested to discriminate between eosinophilc or neutrophilic asthma [3]. There were also no significant differences in breath VOCs between four different sputum inflammatory phenotypes (eosinophil cut-off 3% and neutrophil cut-off of 61% [16]). In a univariate analysis we found 9 VOCs with differences between severe and mild asthmatics

and only one VOC with a difference between high and normal blood eosinophils. After adjusting for multiple testing all respective p-values were > 0.11. Interestingly, the largest difference between moderate and severe asthmatics was found for an unidentified VOC (unadjusted $p<0.001$) that was suspected to be COPD related in a previous study [11].

A recent paper [3] suggests propanol-1 as a potential marker to discriminate between inflammatory phenotypes. Although propanol-1 is known to occur in humans and associated with some diseases and metabolic disorders, propanol-1 is a major part of hand and surface disinfectant and detected in high concentrations in room air of hospital environments. We found no difference of propanol-1 between groups or sputum inflammatory phenotypes [16] in our study (Panel-figure 1.C). The spectrum of compounds associated with asthma is very broad and diverse between studies. A certain overlap between studies appears to exist for alkanes in general, but not for individual alkanes. These as well as other markers or combination of markers suggested to be associated with asthma (reviewed in [1]) were either not among the VOCs that we detected [11] or showed no significant differences between groups. In an effort to find clinically relevant breath VOCs we used a comprehensively characterized asthma cohort and used a breath analysis method that has been benchmarked against others [13], but we were not able to reproduce the positive results of other asthma breath VOC trials.

There is an increasing interest in breath biomarkers [5] but it is important to keep in mind that still no validated VOC biomarker or biomarker pattern exists for any disease (Breath Summit 2019, Loughborough). Despite STARD guidelines, external validation is still rare in breath VOC studies [1] and importantly it is also heterogeneously defined. To evaluate the clinical value of a novel test system the discrimination model from the training patient cohort should be tested in independent patients. Showing that two discrimination models, derived from independent patient groups, result in a similar list of markers [3] or lead to similar clustering of data [2] is a major improvement with respect to independent data validation. However, a true external validation still is missing. The reason for the currently limited success in this field maybe remaining methodological issues or the fact that readily detectable asthma VOC biomarkers do not exist. Despite our high quality standards we can also not exclude that methodological or sensitivity issues are responsible for the non-supportive findings presented here. However, breath biomarkers are not ready for clinical use until all standards are met.

Many valuable insights were gained from the numerous breath VOC studies, especially increasing our awareness for interfering environmental, lifestyle and metabolic factors and for the need of a more standardized methodological approach. Considering these interfering factors it currently appears crucial to identify breath VOCs to be able to assess their origin and biochemical meaning. Databases like PubChem, mVOC or HMDB provide detailed information for VOCs on endogenous production in different species, the relationship to human diseases as well as the occurrence in foods or products potentially playing a role for exogenous exposures. Available standardized collection methods (e.g. ReCIVA breath sampler, Owlstone, UK) and efforts to make collection and analysis methods more comparable between research groups (Peppermint oil consortium) will strengthen research activities that involve multiple centers [3,11] and thereby increase patient numbers and the statistical power – which is desperately needed for a truly external validation.

Yet, at this point, we would like to add a word of caution to the ongoing discussion, as we did not find any significant correlations between VOCs and inflammatory markers in a well-characterized cohort of adult patients with asthma with a broad spectrum of clinical phenotypes.

Olaf Holz*, Benjamin Waschki*, Henrik Watz, Anne Kirsten, Mustafa Abdo, Frauke Pedersen, Markus Weckmann, Oliver Fuchs, Anna-Maria Dittrich, Gesine Hansen, Matthias V. Kopp, Erika v. Mutius, Klaus F. Rabe, Jens M. Hohlfeld§, Thomas Bahmer§, and the ALLIANCE Study Group

*,§ Authors contributed equally

Fraunhofer ITEM, Hannover, Germany , LungenClinic Grosshansdorf GmbH,Grosshansdorf, Germany, Pulmonary Research Institute at LungenClinic Grosshansdorf , Grosshansdorf, Germany, Division of Pediatric Pulmonology and Allergology, University Children's Hospital, Luebeck, Germany, Department of Paediatric Respiratory Medicine, Inselspital, University Children's Hospital of Bern, University of Bern, Bern, Switzerland, Department of Paediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany, Dr von Hauner Children's Hospital, Ludwig Maximilians University, Munich, Germany.

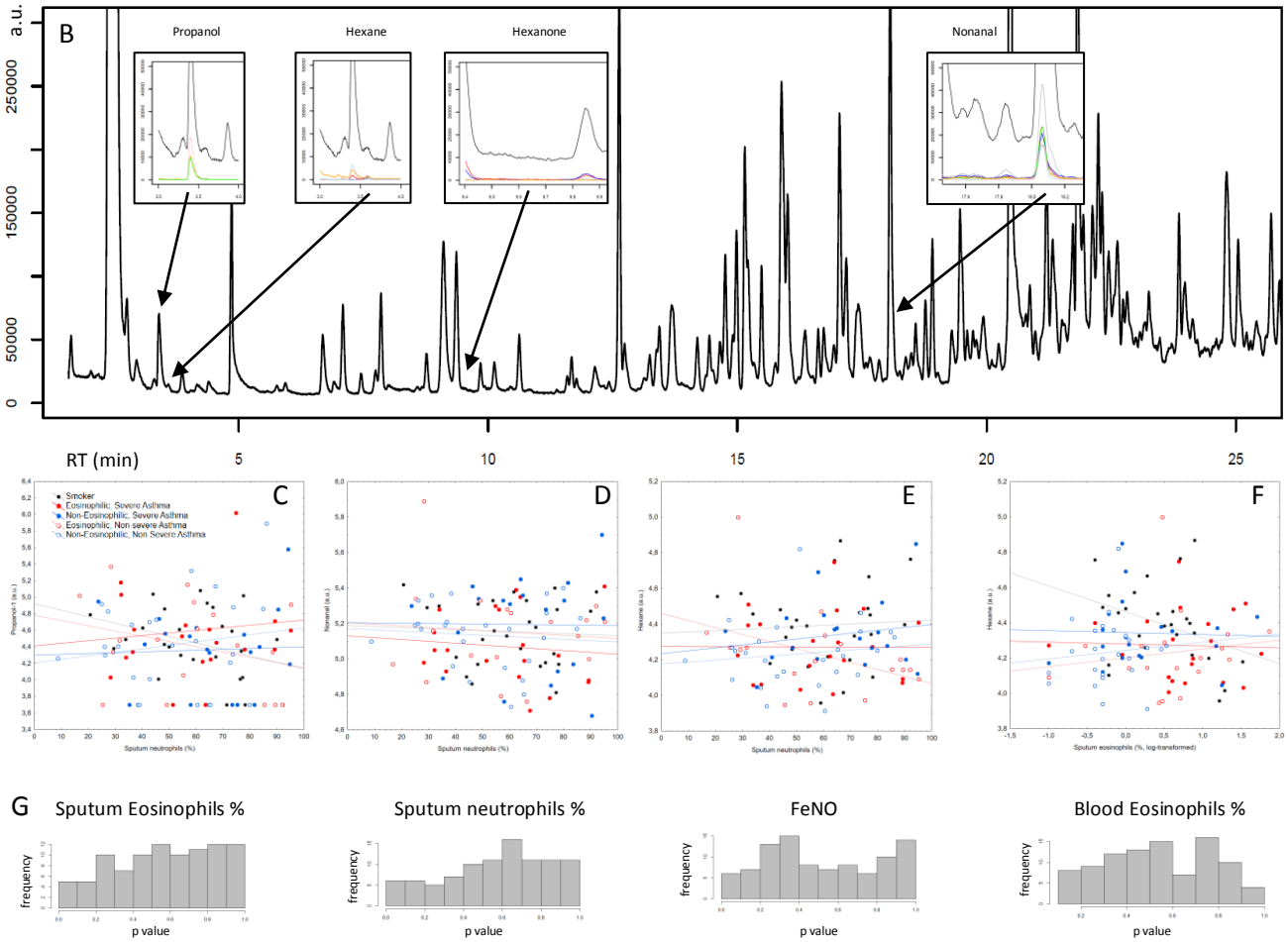
Correspondence: Olaf Holz, Fraunhofer ITEM, Hannover, Germany

Email: olaf.holz@item.fraunhofer.de

Word count: 1302

Multi-Panel Figure 1: A: patient demographics according to asthma phenotypes. Data are presented as median (IQR). Statistics: Kruskal-Wallis-Anova. Abbreviations: BMI, body mass index; PY, Pack-Years; ACT, Asthma Control Test; ICS, inhaled corticosteroids; OCS, oral corticosteroids; % pred., % of predicted; FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; sRtot, specific total airway resistance; R5Hz, resistance at 5Hz; FDRabs, frequency dependence of resistance (resistance at 5Hz – resistance at 20 Hz). & missing values, # Omalizumab or Mepolizomap. **B:** GC/MS chromatogram of one representative patient with inserts and arrows indicating the retention time (RT) for propanol-1, hexane, 2-hexanone and nonanal [3]. The black lines indicates the total ion content (TIC), the insert colored plots show specific masses of the respective VOCs. **C-E:** correlation between VOCs and sputum neutrophils (%). All patients and subgroup correlations not significant (unadjusted p-value >0.05). **F:** correlation between hexane and sputum eosinophils (%). All patients and subgroup correlations not significant (unadjusted p-value >0.05), except for smokers (r = -0.45, unadjusted p value = 0.02, adjusted p value=0.48). Selection of VOCS based on published data by [3]. As shown in **B**, 2-hexanone was not detectable in our samples. **G:** histograms of unadjusted p-values for the correlation between all detected VOCs and sputum neutrophils and eosinophils (%), exhaled NO (FeNO), and blood eosinophils (%). a.u. = arbitrary units

A		Mild to moderate Asthma		Severe Asthma		Smoking asthmatics	p-value
		eosinophilic	non-eosinophilic	eosinophilic	non-eosinophilic		
Number of patients	n	22	31	22	24	34	
Gender	m/f	10/12	19/12	9/13	13/11	11/23	
Age	years	46.0 (38.5; 50.0)	47.0 (34.5; 56.0)	54.0 (50.0; 68.8)	62.0 (53.0; 66.0)	56.5 (49.3; 64.8)	0.002
Height	cm	169.0 (165.2; 176.8)	178.0 (169.0; 182.0)	170.0 (165.2; 176.2)	174.0 (165.5; 178.0)	168.0 (161.0; 175.0)	0.03
BMI	kg/m2	26.3 (22.8; 28.1)	26.3 (23.8; 28.8)	27.7 (25.7; 30.1)	26.1 (23.7; 34.7)	26.7 (23.9; 30.4)	ns
Smoking Status		All patients were Neversmokers or Ex-Smoker < 10 PY				9 Current/25 Ex-Smoker >10 PY	
Cumulative cigarette exposure	PY	2.0 (2.0; 5.8)	3.0 (2.0; 6.0)	2.0(2.0; 4.0)	5.0 (3.0; 5.0)	17.0 (12.3; 40.0)	
Current passive smoke exposure at home	n	2	1	5	2	4 (in Ex-Smokers)	
Exacerbation frequency	never/1/>1 /y	15/4/2[&]	28/2/1	6/4/12	5/5/14	15/7/11[&]	
Asthma Control Test (ACT)		22.0 (17.8; 23.8)	23.0 (20.0; 24.5)	17.0 (14.0; 19.0)	17.0 (14.0; 21.3)	20.0 (15.0; 23.0)	<0.001
ICS use	n/%	17/77%	27/87%	22/100%	24/100%	31/91%	
ICS dose (in fluticason equivalents)	µg	250.0 (125.0; 400.0)	250.0 (50.0; 275.0)	700.0 (500.0; 1000.0)	1000.0 (950.0; 1100.0)	450.0 (200.0; 500.0)	<0.001
Second asthma controller medication	n/%	17/77%	20/65%	22/100%	24/100%	28/82%	
OCS	n	0	0	9	9	7	
OCS dose	mg			10.0 (5.0; 10.0)	10.0 (8.0; 10.0)	5.0 (4.5; 6.5)	
Biologicals#	n	0	1	2	5	3	
Spirometry: FEV1	%pred.	81.0 (75.9; 92.8)	94.4 (84.8; 104.6)	80.2 (67.7; 87.5)	79.0 (53.0; 93.0)	79.7 (65.0; 93.0)	0.006
FVC	%pred.	108.2 (98.6; 118.2)	109.5 (104.3; 116.6)	106.6 (96.7; 112.7)	97.1 (85.0; 112.8)	104.6 (97.7; 114.7)	ns
FEV1/FVC		0.7 (0.6; 0.7)	0.7 (0.7; 0.8)	0.6 (0.5; 0.7)	0.6 (0.5; 0.7)	0.6 (0.5; 0.7)	0.003
Bodyplethysmography: RV/TLC		0.4 (0.3; 0.4)	0.3 (0.3; 0.4)	0.5 (0.4; 0.5)	0.5 (0.4; 0.5)	0.4 (0.4; 0.5)	<0.001
sRtot	kPa*s/L	1.4 (1.0; 1.6)	1.0 (0.8; 1.1)	1.9 (1.1; 2.8)	1.4 (1.1; 2.7)	1.4 (1.2; 2.3)	<0.001
sRtot	%pred.	136.5 (100.5; 155.5)	89.0 (79.7; 110.3)	158.5 (96.9; 249.4)	145.8 (106.3; 265.3)	135.9 (107.2; 225.6)	<0.001
Impulse Oscillometry: R5Hz	kPa*s/L	0.5 (0.3; 0.6)	0.4 (0.3; 0.5)	0.5 (0.4; 0.8)	0.5 (0.4; 0.6)	0.5 (0.4; 0.6)	0.004
FRDabs	kPa*s/L	0.1 (0.1; 0.1)	0.1 (0.0; 0.1)	0.2 (0.1; 0.2)	0.1 (0.1; 0.2)	0.1 (0.1; 0.2)	0.003
FeNO	ppb	26.0 (17.0; 40.0)	19.0 (13.0; 27.0)	29.0 (17.0; 43.0)	24.0 (16.0; 49.0)	16.0 (12.0; 28.0)	0.02
Sputum: Eosinophils	%	3.8 (2.0; 9.2)	0.4 (0.3; 1.0)	5.4 (3.5; 14.1)	0.9 (0.7; 3.4)	3.5 (1.0; 7.1)	<0.001
Neutrophils	%	58.0 (35.1; 82.8)	48.2 (36.3; 66.7)	63.0 (36.8; 69.3)	64.9 (53.1; 80.9)	62.8 (48.2; 75.5)	ns
Blood: Eosinophils	%	6.0 (5.0; 7.8)	3.0 (2.0; 4.0)	7.0 (4.0; 9.0)	2.0 (1.0; 2.0)	3.0 (2.0; 6.0)	<0.001
Eosinophils	Mio/µL	475 (370; 585)	170 (130; 220)	500 (410; 640)	135 (40; 213)	255 (103; 378)	<0.001
Neutrophils	Mio/µL	4455 (3558; 5558)	3340 (3080; 3945)	4300 (3400; 5520)	6775 (3885; 7850)	4100 (2920; 5713)	0.001



References

1. Azim A, Barber C, Dennison P, Riley J, Howarth P (2019) Exhaled volatile organic compounds in adult asthma: a systematic review. The European respiratory journal 54 (3).

2. Vries R de, Dagelet YWF, Spoor P, Snoey E, Jak PMC et al. (2018) Clinical and inflammatory phenotyping by breathomics in chronic airway diseases irrespective of the diagnostic label. The European respiratory journal 51 (1).

3. Schleich FN, Zanella D, Stefanuto P-H, Bessonov K, Smolinska A et al. (2019) Exhaled Volatile Organic Compounds Are Able to Discriminate between Neutrophilic and Eosinophilic Asthma. American journal of respiratory and critical care medicine 200 (4): 444–453.

4. Sterk PJ (2019) Modern Inflammatory Phenotyping of Asthma. Breathomics Is Here to Stay. American journal of respiratory and critical care medicine 200 (4): 405–406.

5. Fowler SJ, Sterk PJ (2019) Breath biomarkers in asthma: we're getting answers, but what are the important questions. The European respiratory journal 54 (3).

6. Ahmed WM, Brinkman P, Weda H, Knobel HH, Xu Y et al. (2018) Methodological considerations for large-scale breath analysis studies: lessons from the U-BIOPRED severe asthma project. Journal of breath research 13 (1): 16001.

7. Global Initiative for asthma. Global strategy for asthma management and prevention 2014. Available: www.ginastma.org.

8. Fuchs O, Bahmer T, Weckmann M, Dittrich A-M, Schaub B et al. (2018) The all age asthma cohort (ALLIANCE) - from early beginnings to chronic disease: a longitudinal cohort study. BMC pulmonary medicine 18 (1): 140.

9. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M et al. (2014) International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. The European respiratory journal 43 (2): 343–373.

10. Pedersen F, Zissler UM, Watz H, Rabe KF, Hohlfeld JM et al. (2019) Rating sputum cell quality in clinical trials for asthma and COPD treatment. International journal of chronic obstructive pulmonary disease 14: 195–198.

11. Gaida A, Holz O, Nell C, Schuchardt S, Lavae-Mokhtari B et al. (2016) A dual center study to compare breath volatile organic compounds from smokers and non-smokers with and without COPD. Journal of breath research 10 (2): 26006.

12. Malásková M, Henderson B, Chellayah PD, Ruzsanyi V, Mochalski P et al. (2019) Proton transfer reaction time-of-flight mass spectrometric measurements of volatile compounds contained in peppermint oil capsules of relevance to real-time pharmacokinetic breath studies. Journal of breath research 13 (4): 46009.

13. Henderson B, Ruszkiewicz D, Wilkinson M, Beauchamp J, Fowler S et al. (2020) A benchmarking protocol for breath analysis: the peppermint experiment. Journal of breath research (14(4)).

14. Lechner M, Moser B, Niederseer D, Karlseder A, Holzknecht B et al. (2006) Gender and age specific differences in exhaled isoprene levels. Respiratory physiology & neurobiology 154 (3): 478–483.

15. Benjamini, Y. and Y. Hochberg (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, Series B. (57): 289–300.

16. Wang F, He XY, Baines KJ, Gunawardhana LP, Simpson JL et al. (2011) Different inflammatory phenotypes in adults and children with acute asthma. The European respiratory journal 38 (3): 567–574.